Amendment) was provided on February 20, 2002. Applicants provide herein a copy of that Supplemental Amendment as Attachment B and believe that this paper satisfies the requirement. Applicants accordingly request reconsideration of the objection.

CLAIM TO PRIORITY

Applicants amend the specification herein by amending the claim to priority. Applicants believe this addresses the Examiner's objection for the form of the claim to priority.

The Examiner has rejected Applicants' claims to priority that predate April 15, 1997. In particular, the Examiner has objected to Applicants' claim to priority to the provisional application U.S.S.N. 60/008/104 (the '104 application), filed on October 30, 1995. A non-provisional application is properly afforded the priority date of a previously filed provisional application when the written description of the provisional application supports the claims of the non-provisional application. The Examiner stated in the March 12, 2002, Office Action that support for sFv-DT390 maintaining CD3 specificity was found as well as support for assays to monitor antibody responses, the inhibitory effects of anti-DT antibodies, and the lack of anti-DT antibody inhibition for SFV-DT390. The Examiner, however, alleges there is a lack of support for claims drawn to methods of inhibiting a rejection response in a primate comprising administering sFv-DT390 so as to reduce the recipient's T-cell lymphocytes by at least 80 percent.

To prove adequate written description in the provisional application, "[I]dentity of description is not necessary." *New Railhead Mfg., L.L.C. v. Vermeer Mfg. Co.*, 298 F.3d 1290, 1296 (Fed. Cir. 2002); accord *Crown Operations Int'l, Ltd. V. Solutia Inc.*, 289 F.3d 1367, 1376 (Fed. Cir. 2002) ("[T]he disclosure as originally filed does not have to provide *in haec verba* support for the claimed subject matter at issue."). Applicants must merely show that the same invention is described. To this end, Applicants respectfully direct the Examiner to at least page 5, lines 18-27; page 13, lines 13-24; page 25, lines 13-25; page 26, lines 7-27; page 27, lines 19-36; page 31, lines 9-19; page 38, lines 16-22, and page 48, line 1, through page 52, line 26, of the '104 application, where the use of sFv-DT390 to reduce T cells is discussed. In particular, on

page 27, lines 19-36, the specification shows that administration of immunotoxin FN18-CRM9 and its human analogs reduced T cells to a average level of less than or equal to 200 cells/mm^3. It is noted that average normal T cell concentrations are well over 1000 cells/mm^3. *See, e.g.*, Neville et al. (J. Immunotherapy 19:85-92) (Attachment C), in which Figure 1 shows a T cell range of 700 to 7000 cells/mm^3 prior to treatment with immunotoxin. Thus there is at least an 80% reduction in T cell lymphocytes as shown in the priority document, and the use of sFv-DT390 as an immunotoxin was clearly contemplated and described in the priority document.

Furthermore, the specification of the '104 application at page 52, line 34, through page 53, line 26, specifically shows that the inventors contemplated toxic effects for sFv-DT390. The *in vitro* toxic effects of sFv-DT390 are discussed as comparable to UCHT1-CRM9 (a human analog for FN18-CRM9). The specification of the '104 application further provides *in vivo* data in monkeys using FN18-CRM9 in Examples 5, 7, and 8. Such a combination of *in vitro* and *in vivo* data should be sufficient to reasonably convey to the artisan that the inventor had possession of the claimed invention at the time of filing. *See* M.P.E.P. § 2163.01. *Cf. Cross v. Iizuka*, 753 F.2d 1040, 1051 (Fed. Cir. 1985) (holding that *in vitro* data can be sufficient to establish a pharmacological utility without clinical data). Adequate written description is present in the '104 application so as to convey that the inventor had possession of the invention claimed in the present application.

Additionally, Examiner alleges that support is similarly lacking in the provisional application U.S.S.N. 60/015,459, filed April 15, 1996. Applicants similarly direct the Examiner to at least page 4, lines 15-28 (which specifically refers to methods of inducing immune tolerance and inhibiting a rejection response comprising exposing the recipient to an immunotoxin that reduces T-cell lymphocytes by 80%); page 6, lines 11-20 (which specifically refers to the sFv-DT390 immunotoxin); page 16, line to page 17, line 10 (which specifically refers to method using immunotoxins comprising an anti-CD3 antibody linked to a diphtheria toxin moiety); page 34, lines 7-18 (which teaches a reduction of T-cells to less than or equal to 200 cells/mm^3, an 80% reduction as described above for the '104 application); and page 49, line 9, through page 50, line 7 (which teaches how to make the sFv-DT390 construct).

In addition, the Examiner is directed to at least page 5, lines 18-27; page 24, lines 13-24; page 25, lines 8-27; page 26, lines 19-36; page 30, lines 12-19, page 38, lines 16-22, and page 48, line 1, through page 52, line 26, in U.S.S.N. 08/739,703, filed on October 29, 1996, in which the sFv-DT390 constructs are also discussed.

Given the support in these applications, Applicants request reconsideration of the priority claim and assert that they are entitled to a priority date of October 30, 1995, for the claimed invention. Reconsideration of the claim to priority is respectfully requested.

DOUBLE PATENTING

The Office Action dated April 5, 2001 rejected claims 1-4, 6, 9-10, and 22 under the judicially created doctrine of obviousness type double patenting as allegedly being unpatentable over claims 1 and 3-8 of U.S. Patent No. 6,103,235 (the '235 patent) in view of Thompson et al. (JBC, 270:28037-41 (1995)).

This double patenting rejection, is overcome, as the application is entitled to the priority date of October 30, 1995, which antedates both the Thompson reference from November 24, 1995 and the '235 patent filed April 15, 1995.

35 U.S.C. § 103

Claims 1-4 are rejected under 35 U.S.C. § 103 as allegedly obvious based on Thompson et al., in view of U.S. Patent No. 5,725,857; claims 1-4, 6, 9-10, and 22 and new claims 26-29 are rejected under 35 U.S.C. § 103 as allegedly obvious based on the same references and further in view of Lu et al. (J. Amer. Soc. Nephrol. 4:1239-1256). As discussed above, however, the present claims have priority to October 1995, which predates the Thompson et al. reference, thereby eliminating Thompson et al. as prior art to this claimed invention. Applicants accordingly request reconsideration and withdrawal of this rejection, as neither of the other references standing alone can render the claims obvious.

35 U.S.C. § 112, ¶ 1

A. Written Description

Claims 1-4, 6, 9, 10 and 22 are rejected under 35 U.S.C. § 112, ¶ 1, for allegedly failing to describe the invention in a way that reasonably conveys to the skilled person that the inventors, at the time the application was filed, had possession of the claimed invention. In particular, examiner alleges that the claims lack written support in the absence of the limitation "by inducing immune tolerance." Applicants respectfully point out the examiner has objected to the exclusion of the phrase "by inducing immune tolerance" based on the written description requirement, while simultaneously asserting that inclusion of the phrase in claims 26-29 results in a lack of enablement. This position, however, seems inconsistent.

Applicants argue below that induction of immune tolerance is indeed enabled by the application. For purposes of this rejection, however, Applicants point out that inducing immune tolerance is an extreme effect in the continuum of inhibiting a rejection response. Lesser effects in the continuum of inhibiting a rejection response include immune suppression. The specification, at least in one embodiment, includes the combined use of immunotoxins and immunosuppressants. The Examiner has argued that the use of immunosuppressants indicates something less than complete immune tolerance is achieved by the immunotoxin – that something less is still inhibition of a rejection response. Assuming, *arguendo*, that immunosuppressants are required in a particular case, then therapy with the immunotoxin alone has merely inhibited a rejection response. Thus, if immune tolerance can be achieved, then the claim to inhibiting a rejection response necessarily has written description. If immune tolerance is not achieved in a particular case and the immunotoxin has merely temporarily removed the need for immunosuppressant or has reduced the need for on-going immunosuppressants, then the claim still has written description as a method of inhibiting a rejection response. Description of inhibiting a rejection response may be found throughout the specification and at least on pages 1, line 14-16, which states that the invention is particularly suited to inhibiting rejection of transplanted organs. Also, page 4, lines 16-18; page 9, lines 1-26; page 33, line 19 through page

34, line 5; page 35, lines 14-18; and page 40, lines 19-26 all refer to inhibiting a rejection response. Applicants respectfully request reconsideration and withdrawal of the objection.

B. Enablement

Claims 26-29 are rejected under 35 U.S.C. § 112, ¶ 1, for allegedly lacking enablement such that a person skilled in the art can practice the invention. In particular, the Examiner has rejected claims 26-29 for allegedly failing to teach one skilled in the art how to inhibit a rejection response in a primate recipient, by inducing immune tolerance. Applicants submit to examiner Thomas et al., *Transplantation* 69, 2497-2508), which is provided as Attachment D and which was provided to the Examiner previously at the interview of September 6, 2001. Thomas et al. unequivocally proves enablement by showing that chronic immunosuppression is not required after a first kidney allograft when the recipient is treated with an immunotoxin and, more significantly, no treatment at all was required with a second donor kidney allograft into an animal treated at the time of the first transplant. This rejection response was specific to the donor, as third party allografts were rejected. Thus, the method of the claimed invention enables one of skill in the art to inhibit a rejection response and to achieve immune tolerance.

Additionally, Applicants respectfully point out that the Examiner has incorrectly associated T-cell recovery with antigen recognition. The cited reference to page 82, lines 6-9 does not make reference to tolerance, only the replenishment of the T cell population following depletion. Tolerance is the lack of ability to recognize an antigen as foreign, not the lack of total T cells. Furthermore, the examiner has pointed to the tables on page 84 and 97 as evidence that tolerance is not achieved. Applicants submit that a review of Table 7 (page 84) clearly shows that all of the animals receiving the FN18-CMR9 immunotoxin had prolonged survival of the graft and with no indication of the use of immunosuppressive agents. In fact, 6 of the 9 had survival beyond the last assay point. The assertion by the Examiner that this table shows the addition of immunosuppressive agents prolonged survival is incorrect.

Applicants respectfully point out that the table on page 97 shows that immunotoxin alone and immunotoxin in combination with immunosuppression result in prolonged graft survival.

The range of survival of group 1 (immunotoxin alone) is equivalent to group 2 (immunotoxin in combination). Group 2 shows survival of the graft at least to day 60 and measured out to at least day 90 post-transplant. Group 1 does has animals that rejected the graft at 51 days; however, there are also animals with graft survival in excess of 165 days. Therefore, the range of group 2 is within the range of group 1. Significantly, even in the case of combination therapy when the immunotoxin was combined with immunosuppressants, the immunosuppression was not chronic but was administered for only three days. See page 97, lines 13-16. Thus, immune tolerance was achieved with either immunotoxin alone or in combination with brief treatment with immunosuppressants.

Additionally, applicants would like to point to the examiner page 87, line 16 through page 88, line 9 where prolonged graft rejection due to tolerance is discussed. A skin graft from the donor animal is not rejected after treatment of the recipient with immunotoxin, although a skin graft from a third party donor was immediately rejected. Such data further supports that the recipient was selectively tolerant to the donor tissue.

Applicants believe this objection is overcome and respectfully request that it be reconsidered and withdrawn.

Pursuant to the above remarks, reconsideration and allowance of the pending application is believed to be warranted. The Examiner is invited and encouraged to directly contact the undersigned if such contact may enhance the efficient prosecution of the application to issue.

Payment in the amount of $1,660.00 ($740.00 RCE fee and $920.00 for Request for Three Month Extension of Time) is to be charged to a credit card and such payment is authorized by the signed, enclosed document entitled: Credit Card Payment Form PTO-2038. It is believed

that no additional fee is required with this submission. However, should a fee be required, the Commissioner is hereby authorized to charge any additional amount or credit any overpayment to Deposit Account No. 14-0629.

Respectfully submitted,

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CERTIFICATE OF EXPRESS MAILING UNDER 37 C.F.R. § 1.10

I hereby certify that this correspondence, including any items indicated as attached or included, is being deposited with the United States Postal Service as Express Mail, Label NoEL924195955US in an envelope addressed to: Box RCE, Commissioner for Patents, Washington, D.C. 20231, on the date indicated below.

Chris Czajka   Date 9/11/02

Attachment A
Marked Up Version of Amended Specification
Pursuant to 37 C.F.R. § 1.121(b)(1)(iii)

The paragraph beginning on page 1, line 6:

--(Thrice amended) This application is a continuation of and claims [priority to] the benefit of U.S.S.N. 08/843,409, filed April 15, 1997, now U.S. Patent No. 6,103,235, which is incorporated herein by reference in its entirety and which is a continuation-in-part of U.S.S.N, 08/739,703 filed October 29, 1996, now abandoned, and claims [priority to] the benefit of U.S. Provisional Patent Application Serial Number 60/015,459, filed April 15, 1996, now abandoned, and U.S. Provisional Patent Application Serial Number 60/008,104, filed October 30, 1995, [1998,] all of which are incorporated herein by reference.--

Attachment B

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Neville et al.	)	
Serial No. 09/383,695	)	Group Art Unit: 1642
Filed: August 26, 1999	)	Examiner: Ungar, S.
For: METHODS OF INDUCING IMMUNE TOLERANCE USING IMMUNOTOXINS	)	

SUPPLEMENTAL AMENDMENT UNDER 37 C.F.R. 1.111

VIA FACSIMILE TRANSMISSION
703-746-3142
ATTN: Examiner S. Ungar
Art Group Unit 1642
Commissioner for Patents
Washington, D.C. 20231

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February 20, 2002

Sir:

The computer readable form in the instant application, Serial No. 09/383,695 is identical with the sequence listing provided by amendment on December 15, 1998, in Application Number 08/737,703, filed October 29, 1996, an application to which the present application claims priority. In accordance with 37 C.F.R. 1.821(e), please use the last filed computer readable form filed in that application as the computer readable form for the instant application. It is understood that the Patent and Trademark Office will make the necessary change in the application number, filing date, and inventors for the computer readable form that will be used for the instant application.

Pursuant to the above amendments and remarks, reconsideration and allowance of the pending claims are believed to be warranted. The Examiner is invited and encouraged to directly contact the undersigned if such contact may enhance the efficient prosecution of the application to issue.

No fee is believed to be due; however, the Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-0629.

Respectfully submitted,

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CERTIFICATE OF FACSIMILE TRANSMISSION

I hereby certify that this correspondence and anything indicated as included with this correspondence is being transmitted via Facsimile No. 703-746-3142, addressed to: Examiner S. Ungar, Group Art Unit 1642, U.S. Patent and Trademark Office, on the date shown below.

Gwendolyn D. Spratt — 2-20-02

Gwendolyn D. Spratt — Date

Attachment C

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7

A New Reagent for the Induction of T-Cell Depletion, Anti-CD3-CRM9

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Summary: We have developed a new reagent for inducing in vivo T-cell depletion and have tested this reagent in rhesus monkeys. The reagent is an anti-CD3ε immunotoxin based on a diphtheria toxin binding-site mutant, CRM9. After administration to monkeys, T cells are depleted from both the blood and lymph node compartments to <1% of their initial values. T-cell depletion is associated with transient immunosuppression, as judged by delayed rejection of RhLA-mismatched skin allografts. T cells are repopulated in both compartments; however, the rate of repopulation is age dependent. The rate is rapid in juvenile animals (12 days) and requires >30 days in old animals. The correlation between repopulation rate and age suggests that the repopulation is thymus dependent and that the repopulated T cells are probably naive T cells. This reagent should be a valuable tool in studying the role of memory T cells in rhesus models of autoimmune diseases and protocols of tolerance induction after organ transplantation. **Key Words:** Immunotoxin—TCR—CD3ε—Rhesus—Transplantation—Autoimmune.

There are no known experimental or therapeutic treatments for inducing peripheral T-cell depletion without affecting other immune system cell types. Radiation kills B cells and bone marrow precursors in addition to T cells (1). Anti-thymocyte/lymphocyte globulin inactivates CD2^{+} immune system precursors (2). Whereas anti-CD3 monoclonal antibody treatment has been shown to induce transient T-cell depletion from the blood compartment, depletion from lymph nodes has not been documented except in rodents, in which very high doses are tolerated (20 mg/kg) and partial depletion has been achieved (3,4). T-cell function and dysfunction is known to be crucial in certain autoimmune diseases and in states of tolerance to foreign major histocompatibility complex (MHC) antigens (2,5,6). T-cell function can be modulated to some degree by drugs that inhibit signaling through the T-cell receptor, such as cyclosporin, or reagents that kill activated T cells, such as methotrexate and interleukin-2 (IL-2) fusion immunotoxin (7). However, no available reagents can specifically eliminate noncycling memory T cells from human and nonhuman primate lymph node compartments (6).

Previously we constructed an immunotoxin directed against human CD3ε based on a diphtheria toxin binding-site mutant CRM9. CRM9 has 1/300 the systemic toxicity of wild-type diphtheria toxin. The immunotoxin, UCHT1-CRM9, killed 3 logs of human T cells (Jurkat) in a nude mouse xenograft system (8). The large therapeutic margin of these

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immunotoxins has been ascribed to the presence of an intact translocation domain (9), as well as optimal intracellular routing mediated by the CD3ε subunit of the T-cell receptor (10).

The efficacy of UCHT1-CRM9 as a T cell–depleting reagent could not undergo preclinical testing in monkeys because rhesus and human anti-CD3ε monoclonal antibodies (mAbs) do not cross-react (11). We therefore have constructed the rhesus analog of UCHT1-CRM9, by using the anti-rhesus CD3ε mAb FN18 (12). FN18 is analogous to UCHT1 and OKT3 in its ability to transduce through the rhesus T-cell receptor (13). We report here that FN18-CRM9 is a highly efficacious reagent for inducing peripheral T-cell depletion in rhesus monkeys.

MATERIALS AND METHODS

Monkey Studies

These studies were carried out at the Biomedical Research Primate Center (BPRC) in Rijswijk and at NIH. Rhesus monkeys (*Macaca mulatta*) were used in protocols approved by the BPRC and the NIMH Animal Care and Use Committee. Animals were prescreened for FN18 T-cell reactivity (11,12) because this epitope is polymorphic in rhesus, 2.7% of unrelated monkeys expressing the negative phenotype (14). Filter-sterilized FN18-CRM9 or FN18 were diluted with 2 ml of phosphate-buffered saline (PBS) and administered as an i.v. bolus on days 0 and 4, days 0 and 2, or on days 0, 1, and 2. Hematology parameters were monitored before the test and just before immunotoxin or FN18 administration on days 0 to 4, and on 7 subsequent days to days 28 through 31. For most monkeys, peripheral blood lymphocytes (PBLs) were isolated from heparinized blood by Ficoll-Hypaque gradient centrifugation (LSM, Litton Bionetics, Kensington, MD, U.S.A.). CD3 subset analysis was performed by using an mAb of mouse origin against rhesus monkey CD3 (FN18; BPRC, Rijiswijk, The Netherlands) in either an indirect or a direct immunofluorescence test with fluorescein isothiocyanate (FITC)-labeled FN18. Staining for CD2, CD4, CD8, and CD20 was performed by using a direct staining method (LEU5B/FITC, LEU3A/PE, LEU2A/PE, LEU16/PE; Becton Dickinson, San Jose, CA, U.S.A.) and then analyzed on a FACScan cytometer (Becton Dickinson) as previously described (11). Calculation of the absolute values of subset numbers was based on white blood cell count and differential and the percentage of positively stained cells by fluorescence-activated cell sorter (FACS) analysis. Lymph nodes obtained by biopsy were macerated, stained, and analyzed by flow cytometry by using the same subset markers used to stain blood. Serum chemistries were monitored before the test and on days 2 and 7. Skin grafting was performed on some monkeys so that each recipient received two grafts from different rhesus donors mismatched at least one *RhLA-A* and *B* loci as determined by a one-stage microtoxicity test using standardized antisera typing reagents (14,15). Two monkeys (Table 1, entries 6 and 7) were studied by FACS analysis after lysis of whole blood. These monkeys were part of a larger group used for evaluation of immunotoxin treatment of experimental allergic encephalomyelitis induced by myelin basic protein. In this method,

TABLE 1. *Summary of monkey data*

Entry	Monkey	Body weight (kg)	Age (years)	Treatment	Dose schedule	Total dose of CRM9 (mg/kg)
1	8629	7.9	6.2	IT	B	0.025
2	2Y	9.5	16.0	IT	B	0.025
3	9135	2.1	1.7	IT	A	0.050
4	B78	1.7	2.1	IT	B	0.080
5	8838	5.1	5.8	IT	A	0.100
6	RQ1225	5.4	6.2	IT	C	0.200
7	RQ964	6.5	4.3	IT	A	0.200
8	MD	7.8	18.2	Ab	B	eqv 0.025
9	C76	2.6	2.5	Ab	B	eqv 0.080
10	B65	5.1	3.9	Ab	A	eqv 0.100

Body weight and age were recorded at the beginning of the experiment.
IT, immunotoxin FN18-CRM9. Ab, FN18 at a mol-equivalent dose of immunotoxin CRM9; A, days 0, 1, 2; B, days 0 and 4; C, days 0 and 2, all equally divided.

blood was centrifuged and the plasma discarded. The packed cells were resuspended with an equal volume of PBS, centrifuged, and resuspended again to eliminate any anti-murine antibodies resulting from immune responses to immunotoxin. Then 100 μl of an appropriate dilution of mAb directly labeled with phycoerythrin (PE) or FITC was added to 100 μl of cell suspension and incubated at room temperature in the dark for 15 to 30 min, and 2 ml of lysing solution (Becton Dickinson) was added. Incubation was continued in the dark at room temperature for 15 min. The cells were pelleted by centrifugation, and the pellet was resuspended in 1 ml of PBS two times before FACS analysis. Stains used were CD20PE (Coulter Corporation, Hialeah, FL, U.S.A.), CD16FITC (AMAC, Inc., Westbrook, ME, U.S.A.), and CD8PE (DAKO Corporation, Carpinteria, CA, U.S.A.). CD3 staining was performed with FITC-FN18 conjugated by Chromaprobe (Mountain View, CA, U.S.A.).

Binding Uptake and Toxicity Studies of Immunotoxins to Monkey and Human Cells

FN18 and UCHT1 were iodinated with Bolton and Hunter reagent (16). Binding to Jurkat cells and selected PBLs was carried out at 0°, and Scatchard analysis was performed as described (16). Uptake was determined by prebinding radiolabeled antibodies to cells on ice, washing, and warming to 37°C. Internalized label was assayed by resistance to pH 2.8–induced dissociation as described (17). Human PBLs were isolated from buffy coats supplied by the NIH Blood Bank by Ficoll (Pharmacia) gradient centrifugation. $CD3^+$ T cells were then isolated by negative selection by using magnetic beads (Dynal, Oslo, Norway) coupled to anti-B cell and anti-monocyte (Leu-12, Leu-16, Leu-M3, and Leu-19; Becton Dickinson) as described (18). The selected cells, which were 95% $CD3^+$ on flow-cytometric analysis, were stimulated with 2 μg/ml phytohemagglutinin (PHA) and cultured in the presence of 20 U/ml IL-2. All experiments were done on cells cultured for ≤2 weeks. Monkey PBLs were purified over Ficoll gradients and cultured overnight in RPMI plus 10% FCS and 20 U/ml IL-2. Nonadherent cells were decanted into plastic flasks coated overnight at 4°C with 25 μg/ml FN18. After 6 days of growth, cells were predominantly $FN18^+$. The cells were exposed to varying doses of the appropriate immunotoxin for either 5 or 20 h and pulsed with ^{3}H-L-leucine for the final hour to measure the amount of inhibition of protein synthesis relative to a non-treatment control, as previously described (16).

Immunotoxin Construction

The hybridoma secreting UCHT1 was kindly provided by Dr. Peter Beverley, Imperial Cancer Research Fund, and was grown in ascites fluid and purified over immobilized protein A. This immunoglobulin (Ig)G1 is directed at the CD3ε conformational epitope of human CD3 (19). FN18, also an IgG1, is the rhesus analogue of UCHT1 and shares with it the property of being a T-cell mitogen in the presence of mixed mononuclear cells (12). These two antibodies show no cross-reactivity (12). FN18 was produced in hollow fiber and purified over protein A. The strain of *Corynebacterium diphtheriae* used for production of CRM9, C7 ($\beta^{h\ tox\text{-}201\ tox\text{-}9\ h'}$) was obtained from Dr. R. Holmes, Uniformed Services University of Health Sciences, Bethesda, MD, U.S.A. (20). CRM9 was recovered from the supernatant of 30-L fermentation runs under careful control of iron concentration (21) and purified as described (22). Immunotoxins were synthesized as previously described by thiolating both the mAb moiety and the toxin moiety and then cross-linking with bismaleimidohexane (23). Large-scale purification of immunotoxin was accomplished by high-pressure liquid chromatography (HPLC) size-exclusion chromatography on MODcol (St. Louis, MO, U.S.A.) 2-inch × 10-inch column packed with Zorbax (DuPont Company, Wilmington, DE) GF-250 6 μm, 150 Å. Fractions containing 1:1 toxin to antibody mol ratios were isolated for these studies.

Detection of Anti-DT Antibodies

An enzyme-linked immunosorbent assay (ELISA) was used. CRM9, 10 μg/ml, was adsorbed to Costar 96-well EIA/RIA flat-bottom plates (Costar; Cambridge, MA, U.S.A.), washed with PBS containing 0.1% Tween 20, blocked with 3% gelatin, and again washed before adding 100 μl of appropriately diluted monkey or human sera. Development used protein A/G-alkaline phosphatase (Pierce, Rockford, IL, U.S.A.).

RESULTS

Immunotoxin-Induced T-Cell Depletion and Repopulation in Blood

In Fig. 1, left, $CD3^+$ blood T-cell counts are shown for seven animals treated with varying doses

of immunotoxin over a 4-day period (see Table I for particulars on each monkey). T cells remained depressed to <500 cells/mm^3 after the last dose of immunotoxin between 2 and >28 days. Treatment with antibody alone depresses blood T cells for only 1 day after the last antibody dose (Fig. 1, right). Inspection of the data reveals that after immunotoxin-induced T-cell depletion, repopulation in the blood is highly variable and is age dependent. This is shown in Fig. 2, in which the time to reach 500 CD3$^+$ cells/mm^3 is plotted versus monkey age (Fig. 2, left). The dashed line is a linear curve fit with R = 0.95. In Fig. 2, right, the time taken to repopulate CD4$^+$ cells to 300 CD3$^+$ cells/mm^3 is shown, R = 0.94. At the last day of data collection, six of seven animals had repopulated both CD4$^+$ and CD8$^+$ T-cell subsets. The median CD4$^+$/CD3$^+$ ratio was 0.44 compared with 0.52 before treatment. One animal failed to achieve observable CD4$^+$ repopulation (entry 6).

Most immunotoxin-treated animals exhibited an increase of CD2$^+$ and CD8$^+$ cells in excess of CD3$^+$ cells early in the repopulation phase. Two animals were therefore monitored for CD16 expression (Fig. 3). CD16 expression peaked between days 8 and 15 and exceeded 1,000 cells/mm^3, which at this time was in great excess over the CD3$^+$ cell population. CD8 expression mirrored CD16 expression over this period, indicating that generation of NK cells accounted for these findings. NK cells become a significant proportion of mononuclear cells in humans after autologous bone marrow transplantation (24).

Immunotoxin-Induced T-Cell Depletion in Lymph Nodes

Depletion of T cells in the lymph nodes was monitored by analyzing changes in the percentage of CD3$^+$ cells and CD20$^+$ cells on biopsies of inguinal/axillary nodes that were macerated, stained, and then subjected to flow cytometry. The immunotoxin treatment produced a marked reduction in the percentage of total lymph node cells staining positive for CD3 (Fig. 4, left). At the same time, the fraction of cells staining positive for the B-cell marker dramatically increased (see Fig. 4 legend). This is consistent with an immunotoxin-induced specific depletion of T cells. Only small changes were noted in the antibody-treated animals (Fig. 4, right). At the highest dose of immunotoxin used, T-cell depletion in the lymph node was more pronounced, CD3$^+$ cells decreasing to 0.25% of the total cells 2 days after the last immunotoxin treatment (entry 7).

Immunotoxin-Induced Prolongation of Allograft Survival

Two monkeys (entries 5 and 10 in Table I) were grafted with skin from two different donors mismatched at A or B MHC class I loci. One received FN18-CRM9 and the other received 1 mol equivalent of FN18. The dates of graft rejection were noted to assess immunotoxin-induced immunosuppression. Ten saline-treated controls (not listed in Table I) rejected at 9.2 ± 0.2 days. The immunotoxin-treated animal exhibited a marked prolongation of graft survival times lasting 19 and 20 days, averaging 10.3 days longer than the saline-treated controls. The antibody-treated animal exhibited a slight increase in graft survival, both grafts rejecting at day 12.

Indicators of Immunotoxin Systemic Toxicity

The administration of FN18-CRM9 was well tolerated. The only noticeable untoward effect was generalized or facial erythema noted within 15 min after the infusion in some animals. This gradually subsided over the next 7 days. Animals ate well and maintained weight. Clinical chemistries in animals

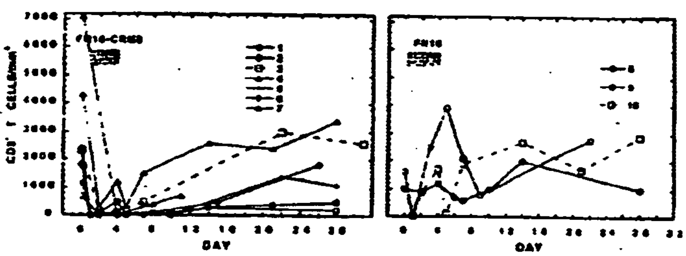

FIG. 1. Blood T cells (CD3$^+$) were enumerated by flow cytometry before and after immunotoxin treatment (left), or FN18 antibody alone (right). Maximum treatment duration is delineated by bars. Numbers beside graph symbols refer to individual monkeys listed in Table 1, where dose, treatment schedule, and monkey age are listed. For graphic purposes, pretreatment data that ranged from − 10 days to day 0 are shown on day 0.

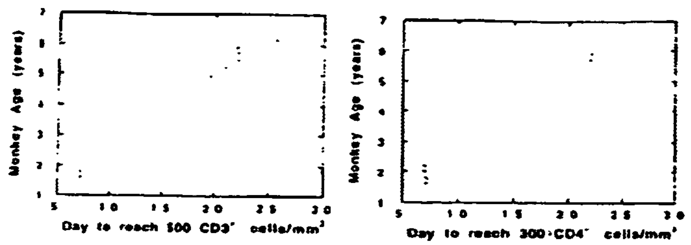

FIG. 2. The time to repopulate blood T cells after immunotoxin-induced depletion is plotted as a function of monkey age. The dashed line is a least-squares fit of the data. Left, repopulation of CD3$^+$ cells to a value of 500 cells/mm^3, $R = 0.95$. Right, repopulation of CD4$^+$ cells to a value of 300 cells/mm^3, $R = 0.94$. All monkeys shown in Fig. 1, Left are included, with the exception of entry 2, the oldest monkey, which never repopulated >200 CD3$^+$ cells/mm^3 during the study period, and entry 6, which failed to repopulate CD4$^+$ cells during the study period.

treated with 0.05 mg/kg immunotoxin were normal except for a transient elevation of lactate dehydrogenase (LDH), 1.4 times the upper limit of normal (ULN) at day 2. At doses of 0.1 mg/kg, serum transaminases were also elevated up to 3.5 × ULN (data not shown).

Primary and Secondary Antibody Responses During T-Cell Depletion

The changes in antitoxin titers after immunotoxin administration are shown in Fig. 5. The kinetics of titer increase are typical for a primary response for monkey 8838, with the first significant titer increases noted 7 days after immunotoxin. All of the monkeys treated with immunotoxin and listed in Table 1 were selected for their lack of preexisting antitoxin titers. However, some monkeys have titers to diphtheria toxin (DT) in the absence of a history of toxoid administration. These titers are presumably acquired from infections with toxigenic strains of *C. diphtheriae*. Two such monkeys are 1WS and C81, and steep increases in titers are observed after day 3 of immunotoxin exposure (Fig. 5). Monkey 1WS received immunotoxin (25 μg/kg total) on day 0 and 4. Blood T-cell depletion was seen on days 1 and 2, and partial repopulation occurred on day 4. However, the day 4 immunotoxin dose was without observable effect (data not shown) in contrast to those of antitoxin-negative monkeys treated with this dose schedule (see Table 1 and Fig. 1). Monkey C81 received immunotoxin (100 μg/kg total) on days 1, 2, and 3, before the secondary response occurred. In addition, this monkey received a 100-fold excess of CRM197, a nontoxic DT mutant capable of adsorbing antitoxin in in vitro assays (25). The monkey experienced marked blood T-cell depletion (data not shown) and depletion of lymph node T cells from 75 to 17%. In spite of this degree of T-cell depletion, a marked secondary response took place. We have not yet

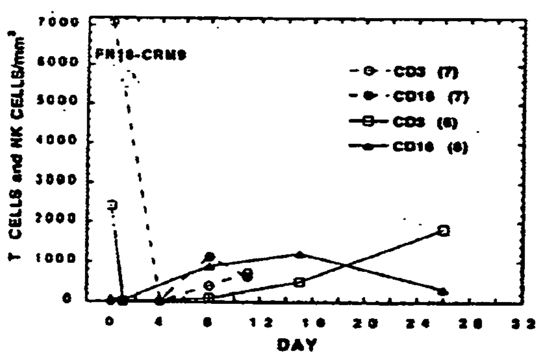

FIG. 3. Blood T cells (CD3$^+$) and natural killer (NK) cells (CD16$^+$) were enumerated by flow cytometry after immunotoxin-induced T-cell depletion. NK cells increased to high values in the early postdepletion period and exceeded the levels of T cells.

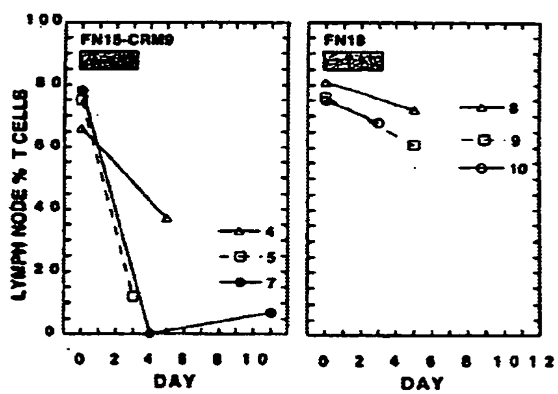

FIG. 4. Lymph nodes were obtained by biopsy before and after immunotoxin treatment, Left, or antibody treatment, Right. Nodes were macerated and the percentages of T cells (CD3$^+$) and of B cells (CD20$^+$) were enumerated by flow cytometry. The percentage of remaining T cells is correlated with the amount of immunotoxin given and reached 0.25% at 0.2 mg/kg (entry 7; see Table 1). In the immunotoxin-treated nodes, the percentage of B cells ranged from 8 to 46% before treatment to 61 to 86% after treatment. B/T cell ratios were unchanged by antibody treatment.

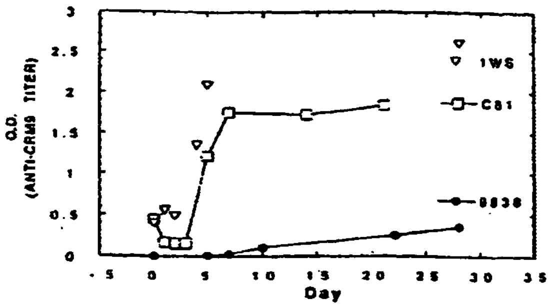

FIG. 5. Enzyme-linked immunosorbent assay (ELISA) was used to quantify rhesus antibody titer to CRM9 before (day 0) and after administration of FN18-CRM9 immunotoxin initiated after sera collection on day 0. ELISA was performed at 1:100 dilution of sera. Monkey 8838 had no detectable preexisting titer, and the subsequent increase is typical of a primary response. Monkeys 1WS and C81 had preexisting titers and exhibited classic secondary responses that were first detected in sera at day 4. Monkey C81 also received an excess of CRM197, a nontoxic DT mutant that can absorb antitoxin in vitro (see Results). Monkey C81 received immunotoxin and CRM197 on days 0, 1, and 2 before the secondary response became manifest.

evaluated primary and secondary antibody responses in monkeys that received 200 μg/kg and had lymph node T-cell depletion to <1%.

Comparison of Binding and T-Cell Toxicity Parameters for Anti-Human and Anti-Rhesus-CD3 Antibodies and Immunotoxins

Because anti-CD3-CRM9 may have clinical utility, it was of interest to compare the receptor binding and uptake of the anti-human antibody, UCHT1, and the anti-rhesus antibody FN18. The comparisons were done on isolated CD3 selected T cells from human and rhesus buffy coats. Labeled antibody binding and uptake were comparable, as was immunotoxin toxicity measured by protein-synthesis inhibition (Table 2). All of the cell types exhibited a rapid internalization process under conditions designed to minimize any Fc receptor interactions.

DISCUSSION

The data presented in Figs. 1 through 3 show that the anti-CD3 immunotoxin is very effective at depleting blood compartment T cells, these decreasing to ≤1% of their pretreatment values. However, because the blood contains at most 5% of the total body T cells (26), we also assayed the lymph nodes. Here we could not measure absolute values, but only the percentage of T cells and B cells that revealed a marked posttreatment inversion of the usual T/B cell ratio. The absolute reduction in T cells in the lymph node is likely to be much greater than the fractional reduction because the absolute number of B cells in the node appears to be relatively unchanged by the anti-CD3 immunotoxin. By increasing the immunotoxin dose to 0.2 mg/kg given in two or three divided doses, we were able to decrease the lymph node T cells to <1% of the total lymph node cells. Subsequent studies in rhesus monkeys have confirmed this finding (S. Knechtle et al., unpublished data). Because most of the T cells in a normal node are resting T cells, the immunotoxin is reducing this population. This is a unique attribute of anti-CD3-CRM9. Other anti-T cell immunotoxins show only small reductions of blood compartment resting T cells (27,28). The only treatments capable of reducing resting T cells to this level are total body irradiation or lymphoid irradiation plus anti-lymphocytic globulin. However, these treatments also reduce B cells and stem cell populations (1).

After the last immunotoxin dose in the entry 5 monkey, T cells remained <100 cells/mm^3 (Fig. 1). This delay in repopulation was associated with delays of 9.7 and 10.7 days in the two skin allograft rejections beyond the saline controls. Although we

TABLE 2. *Comparison of anti-human and anti-rhesus anti-CD3ε antibodies and immunotoxins on T cells*

	Internalization		Receptor		
Cells	Bound (%)	$t_{1/2}$ (min)	Number (cell^{-1})	Affinity (M^{-1})	Immunotoxin ID$_{50}$ (M)^a
Human PBLs	13	5	2×10^4	2×10^8	2×10^{-11}
Monkey PBLs	19	7.5	2×10^4	1×10^8	3×10^{-11}

ID$_{50}$, median infective dose; PBL, peripheral blood lymphocyte.
^{a} Measured at 20 h.

report on only two allografts in one immunotoxin-treated recipient, the data are highly significant when compared with those of the saline controls and the antibody-treated animal. It thus appears that immunotoxin-induced T-cell depletion resulted in a temporary loss of a classic T cell–mediated function. However, in this case, the repopulated cells were fully competent to elicit skin-graft rejection.

Recently FN18-CRM9 at higher doses has been reported to induce long-term tolerance to mismatched rhesus kidney allografts (S. Knechtle, C. Graeb, D. Neville, N. Hanaway, D. Watkins, E. Geissler, and J. Wang, abstract European Society for Organ Transplantation Esot '95, Oct. 3–7, 1995, Vienna/Austria).

Anti-CD3 antibodies can deplete T cells from the blood (29,30), cause T-cell activation (31,32), and induce immunosuppression (29,31). The doses used in this study are smaller than those usually employed to achieve these effects, and our FN18 controls show that our immunotoxin effects are toxin mediated. However, the role of conjugate anti-CD3 interactions with monocyte and B-cell Fc receptors in the in vivo immunotoxin intoxication process remains to be explored.

T-cell repopulation after immunotoxin was found to be highly correlated with monkey age (Fig. 2), which is also correlated with thymic function. Thymic involution in rhesus monkeys begins generally after 3 years is reached. The correlation between T-cell repopulation and age strongly suggests that repopulation occurs through the thymus in young monkeys (33). Because recent thymic emigrants to the periphery are known to be naive T cells (34), it is possible that a round of T-cell depletion and repopulation will have the effect of lowering the ratio of memory to naive T cells and might achieve erasure or partial erasure of T-cell memory. However, the repopulation of the naive $CD4^+/CD45RA^+$ subset is slow after T-cell ablation by chemotherapy, and repopulation is believed to be dependent on residual thymic function (33,35). $CD4^+$ T cells are involved in both acute allograft rejection and long-term suppression of allograft rejection (36). Therefore, it is not at all clear what the effects will be of a round of immunotoxin-induced T-cell depletion and repopulation with respect to potential autoreactive and suppressor T-cell functions. In particular, it will be of interest to determine what the mechanistic effects are of transient T-cell depletion and repopulation on rhesus models of T-cell–driven autoimmune diseases and on the processes of induction of allograft transplantation tolerance.

In the past, anti-lymphocyte/thymocyte globulin has been used to induce transient T-cell depletion for the treatment of autoimmune diseases (37) and in animal models of transplant tolerance (2,6). Because anti-CD3-CRM9 appears to be well tolerated, it may offer clinical advantages over anti-lymphocyte/thymocyte globulin, especially with respect to T-cell specificity and the magnitude of T-cell depletion. In addition, anti-CD3-CRM9 may be useful in the treatment of graft-versus-host disease, acute allograft rejections, and $CD3^+$ cutaneous T-cell lymphomas.

The monkeys used in this study were selected to be free of measurable anti-diphtheria toxin antibodies at 1:100 serum dilution. High rhesus antitoxin titers apparently acquired from infections with toxigenic strains of *C. diphtheriae* can block the effects of FN18-CRM9 on T cells in vivo and in vitro (25). Human antitoxin titers, compared to those of rhesus, are relatively inefficient in blocking anti-human-CD3-CRM9 toxicity (J. Thompson and D. Neville, unpublished data). In addition, the human blocking antibodies are directed largely at the C terminus of the toxin, which has been eliminated in an engineered fusion protein version of this immunotoxin (25). This should ensure good efficacy of engineered anti-CD3-immunotoxins in spite of anti-DT titers in human subjects.

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Attachment D

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DURABLE DONOR-SPECIFIC T AND B CELL TOLERANCE IN RHESUS MACAQUES INDUCED WITH PERITRANSPLANTATION ANTI-CD3 IMMUNOTOXIN AND DEOXYSPERGUALIN

ABSENCE OF CHRONIC ALLOGRAFT NEPHROPATHY[1]

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Tolerance induction can prevent acute kidney allograft rejection without chronic immunosuppression. It is uncertain whether specific tolerance can prevent chronic allograft nephropathy (CAN), which involves both nonimmune and immune injury. This report provides evidence that immunologically tolerant macaques, induced with immunotoxin and deoxyspergualin, developed neither acute rejection nor CAN. Long survivors, bearing MHC-mismatched grafts without chronic immunosuppression for 0.8 to 3.4 years, exhibited general immune competence with donor-specific T and B cell tolerance and no functional or histological evidence of CAN. Stringent criteria for tolerance were satisfied by specific prolongation of donor skin grafts with rapid rejection of third-party skin, followed by indefinite acceptance of a second donor kidney graft and establishment of microchimerism. Primate tolerance with documented absence of CAN may give impetus to the clinical application of tolerance.

Current immunosuppressive management for human kidney transplantation has advanced such that early graft loss to acute rejection is uncommon. In contrast, graft loss to chronic rejection, i.e., chronic allograft nephropathy (CAN), has not improved substantially for more than a decade (*1*). Although CAN is the leading obstacle to long-term (LT) kidney transplant success, its pathogenesis is poorly understood (*2*). Induced by inflammatory, alloimmune, and donor tissue injuries (*3–5*), CAN is not effectively curtailed by chronic immunosuppression (CI), and, in fact, may be exacerbated by CI-related nephrotoxicity. Moreover, the severe donor organ shortage, compounded by increasing numbers of end-stage renal disease patients, is aggravated by increasing requirements for secondary transplants largely due to CAN. Thus, a need exists for new approaches to prolong allograft survival without CAN.

Multiple immunological and nonimmunological factors and injuries are implicated in the process of CAN (*3*). However, compelling evidence suggests that alloimmunity plays a vital role in the process (*2, 6*). Furthermore, nonimmune graft injury can provoke alloimmune responses. For example, short-lived dendritic cells (DC), the antigen-presenting cells that most effectively stimulate T- and B-cell responses, are preferentially activated by ingesting apoptotic vesicles from injured donor cells (*7*). Therefore, it is conceivable that deliberate immunological tolerance induction can contribute to solving the quandary of CAN.

Several tolerance induction strategies have been successfully applied to induce kidney allograft acceptance in nonhuman primates without CI (*8–11*), providing impetus for clinical tolerance application (*12*). Although primate models represent the essential preclinical step for translation to human tolerance, evidence for true MHC-specific tolerance with absence of CAN in LT primate transplant recipients has not been convincing (*13*).

This follow-up report extends our recent study using a unique day of transplant treatment to reproducibly promote LT, stable tolerance to MHC-incompatible primate kidney allografts. The strategy involves brief treatment with anti-CD3ε-mutant diphtheria toxin-immunotoxin (IT) and deoxyspergualin (DSG) (*14, 15*). In this paradigm, a quiescent window is created by combining the highly effective T cell-ablating properties of IT to eliminate lymph node T cells (*16*) with DSG's blockade of nuclear factor (NF)-κB-dependent nuclear translocation, and thus proinflammatory cytokine production (*14*) and DC maturation (*15*).

Within this window, a reorganizing, T cell-deficient immune system reshapes itself without "danger" (*17*) to promote donor-specific hyporesponsiveness. The early posttransplantation environment, dominated by T helper 2 cytokines and lacking both mature lymph node DC and T cells, induces stable tolerance without irradiation, splenectomy, chimerism, or CI (*15*). The data here present definitive evidence showing that 12 tolerant, LT primate recipients with specified MHC class I and II allele mismatches (268 days to >3.5 years) did not develop CAN. Furthermore, the data reveal a uniform absence of alloantibodies with normal IgG responses to unrelated exogenous antigens, indicating specific B cell tolerance. The results suggest both acute rejection and CAN are eliminated by T and B cell tolerance

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induction, making a persuasive case for the clinical application of tolerance.

MATERIALS AND METHODS

Animals. After a 60-day quarantine, normal 3-kg male rhesus macaques (*Macacca mulatta*) were maintained in a restricted facility. The animals, obtained from Covance (Alice, TX) and LABS (Yemassee, SC), were free of known pathogens and negative for diphtheria toxin antibodies (Ab) as specified (*16*). Procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Primates under supervision of the Institutional Animal Care and Use Committee. For routine handling, animals were sedated with 10 mg/kg i.m. ketamine.

Histocompatibility testing. Pretransplantation testing guaranteed recipient-donor MHC class I and II incompatibilities. DNA typing of Mamu MHC alleles was performed by polymerase chain reaction (PCR) as described (*18*) using a panel of *Mamu* class II DR and *Mamu* class I A locus allele sequence-specific primers (SSP), the fidelity of which was verified by sequencing.

Transplantation and supportive care. Heterotopic kidney allotransplantation with intrinsic bilateral nephrectomy established a working allograft model. Serum creatinine and blood urea nitrogen (BUN), creatinine clearance, and urinary protein levels were assayed by the UAB Hospital Outreach Laboratory. Postoperative antibiotics and posttransplantation analgesia and nutritional support were administered in the first weeks (*14–16*). In two LT survivors, a second kidney transplant from the original donor was placed on the contralateral side 14–21 months after the first transplantation.

Experimental groups and immunosuppression. The tolerance induction, described in detail elsewhere (*14*), was initiated 4 hr before transplantation and consisted of IT given on day 0 and +1, DSG (Nippon Kayuka, Tokyo, Japan) given daily on days 0–14, and a taper course of methylprednisolone (Upjohn, Kalamzoo, MI) given during IT administration. The methylprednisolone was given at 7 mg/kg on day 0, 3.5 mg/kg on day +1, and 0.35 mg/kg on day +2. No other immunosuppressive agents were used. Two recipients were given a modified protocol by infusion on days 2–4 after transplantation with isolated donor peripheral blood CD34$^+$ cells (obtained after mobilization with granulocyte colony-stimulating factor; Amgen, Thousand Oaks, CA). These recipients were included because, like other DSG-treated recipients, they failed to exhibit chimerism.

Kidney biopsies. Open kidney transplant biopsy specimens were fixed in 10% buffered formalin and stained with hematoxylin and eosin. A renal transplant pathologist, without knowledge of the protocol, assigned a Banff classification of allograft pathology (*19*). Immunohistochemical analysis was performed on frozen 5-mm sections after fixation in acetone. Staining with Ab to transforming growth factor (TGF)-β1 or fibroblast growth factor (FGF)-2 (Santa Cruz Biotechnology, Santa Cruz, CA) was developed using a Dako streptavidin-biotin kit (Dako Corporation, Carpenteria, CA).

Microchimerism. The Mamu-DR PCR-SSP typing method was adapted for detecting donor DNA in recipient blood by a method with sensitivity of 0.05–0.005% as previously described (*18*). We tested simultaneously for two DRB-mismatched donor-specific alleles because of variable sensitivity of detecting individual donor alleles.

Flow cytometry analysis. Alloantibody analysis was done by flow cytometry, using recipient serum and donor peripheral blood lymphocytes. Bound IgM and IgG were detected with respective fluorescein isothiocyanate-conjugated goat anti-human isotype-specific Abs (Jackson ImmunoResearch, West Grove, PA). T cells were identified with biotinylated anti-CD3ϵ (BioSource, Camarillo, CA) plus PerCP streptavidin (Becton Dickinson, Palo Alto, CA) and B cells with anti-CD20 (Becton Dickinson).

ELISA. Abs to IT-related antigens, diphtheria toxin (provided by DMN, NIMH), mouse IgG1 (MOPC 21; Sigma), and mouse IgG1 FN18 monoclonal antibody (mAb; BioSource) were tested by ELISA. Microtiter plates (Costar Corp., Acton, MA) were coated with the specific antigens, and bound Abs were detected with a protein A-alkaline phosphatase conjugate (Sigma). Chromogenic products were developed using a Pierce Alkaline Phospatase Kit (Pierce Chemical Co., Rockford, IL), and products were measured in a BioRad ELISA plate reader at 405 nm (BioRad, Redwood, CA).

RESULTS

Histocompatibility profile of LT kidney allograft survivors induced with peritransplantation IT plus DSG. The data in Table 1 list the LT recipient-donor pairs and their multiple DR mismatches. Additional molecular typing for the most

TABLE 1. Immunosuppressive treatment and class I and II MHC allele mismatches per donors/recipients

Recipient	Immunosuppression	Days after Tx	Class I *Mamu-A** locus mismatched alleles[a]	*Mamu-DRB1** mismatched alleles[a]	*Mamu-DRB* non-B1 mismatched alleles[a]
TIJ	IT+DSG	>1292	03	0303; 1005/7	[B3*0402/5], [B*w601]$^+$, [B*w201]
T4P	IT+DSG	>1220	02	0403; 1002/4	[B3*0403], [B5*0304], [B5*0404], [B*w501], [w602]
AXE	IT+DSG	>1270	03	0303	[B*w101]
95C137	IT+DSG	>806	03; 06	1002/4	[B*w201]
CFB[b]	IT+DSG	>820	0	0405; 1002	[B3*0403/4], [5*0301/2], [B*w601]$^+$
CHE[b]	IT+DSG	>861	02	0309; 0403	[B*w201], [B*w303], [B*w401], [B*w702]
DAC	IT+DSG	268	04	0309; 1006	[B*w201], [B*w304]$^+$, [B*w702]
96C176	$F(ab)_2$IT+DSG	>462	03	0404; 1006	[B3*0403], [B5*0303], [B*w501], [B*w101/2], [B*w604/6], [B*w601]
96C044	$F(ab)_2$IT+DSG	>462	03	0402	[B5*0301/2], [B*w201]
95D471	$F(ab)_2$IT+DSG	>435	0	1006; 0305/6/10	[B5*0304], [B*w101], [B*w201], [B*w602], [B*w601]$^+$, [B*w604/6]
97D141	$F(ab)_2$IT+DSG	>356	02; 04	0305/6/10	[B3*0402/5], [B5*0304], [B*w101], [B*w307]$^+$, [B*w602], [B*w601]$^+$, [B*w604/6]
97D389	$F(ab)_2$IT+DSG	>322	04; 07	0305/6/10, 1006	[B3*0402/5], [B*w101], [B*w304]$^+$, [B*w702]
95C163	IT	676	04; 05	0305/6/10	[B5*0304], [B*w307]$^+$; [B*w601]$^+$, [B*w602], [B*w604/6]

[a] The data shown represent mismatched donor alleles per donor/recipient combination. Class I *Mamu-A* locus and class II *Mamu-DRB* typing was performed by PCR-SSP with allele-specific primers.

[b] Donor stem cells infused into these two recipients on day 0 and +1 without establishment of demonstrable chimerism.

$^+$ Represent an allele cluster.

common MHC class IA locus alleles demonstrated class I incompatibilities in 11 of 13 combinations. The absence of measurable class I incompatibility in the remaining 2 of 13 recipients does not indicate a class I match, because the SSP panel included only a partial number of A locus alleles and no B locus alleles. The documented MHC incompatibilities, particularly at DRB, allow inference that durable LT graft acceptance was attributable to induction treatment.

Rejection-free status with normal renal function in the long survivors treated with IT plus DSG. This report focuses only on the LT survivors listed in Table 1 at 268 days to >3.5 years after transplantation. Our intent is only to provide documentation for the uniform absence of CAN and the evidence for specific B cell tolerance after successful induction with IT plus DSG. Of note, we recently reported that peritransplantation IT plus DSG induction without blood or bone marrow cell infusion can promote rejection-free survival in the absence of any additional immunosuppression in a majority of recipients (*15*). With intact IT (FN18-CRM9) plus the 2-week DSG treatment course, we have reported 80% (4/5) rejection-free survival, whereas with an abbreviated 5-day DSG treatment course, rejection-free survival was reduced to 50% (2/4). Thus, IT plus DSG synergy is favored by continuing the DSG treatment for 2 weeks.

Among the LT recipients listed in Table 1, seven received IT as the intact IgG conjugate and five received $F(Ab)_2IT$. The combination of DSG and $F(Ab)_2IT$ has proven to be unusually effective, evoking no systemic proinflammatory cytokine release and yielding 100% rejection free, LT survival without morbidity or mortality in 5/5 recipients. Additional recipients have shown precisely the same trend, but, because their follow up is too early (200–250 days) for confident assessment of chronic rejection and B cell tolerance, they were not included in this report. (Thomas J, in preparation). All the LT survivors listed in Table 1 have maintained excellent renal allograft function, gained weight and are rejection free at a current median 641 days without any CI. One exception, DAC, died accidentally of food aspiration at 9 months after transplantation with normal graft function and histology.

Importantly, when initiated on the day of transplantation, neither IT alone (*16, 20*), $F(Ab)_2IT$ alone (*15*), nor DSG without IT (*14*) promotes stable tolerance in rhesus macaques. A single recipient (95C163) was an LT survivor after induction with high-dose IT (300 μg/kg) alone. This recipient eventually succumbed to CAN and was included to provide a comparison for laboratory findings in the tolerant recipients given IT plus DSG. This animal, who received IT without DSG, showed deteriorating graft function by 300 days after transplantation.

Of note, two animals (CHE and CFB) received a modified treatment with IT, DSG, and isolated donor $CD34^+$ cells that were infused on days 2–4 after transplantation. Because these two LT survivors failed to demonstrate measurable chimerism (blood, bone marrow, skin or lymph nodes), they were included. Seven other recipients treated with donor $CD34^+$ cells after transplantation (not included) developed progressive wasting disease and/or gastrointestinal abnormalities. This deleterious outcome, seen only after donor $CD34^+$ cell infusion, was associated with focal hepatic, splenic, and mesenteric microchimerism. Since mature T cells were present as contaminants in the $CD34^+$ cell suspension, this complication may reflect chronic graft-versus-host disease.[5] In any case, administration of peripheral donor $CD34^+$ cells after IT clearance may constitute an unacceptable risk in primate preclinical testing and should probably be avoided in clinical trials of this tolerance strategy.

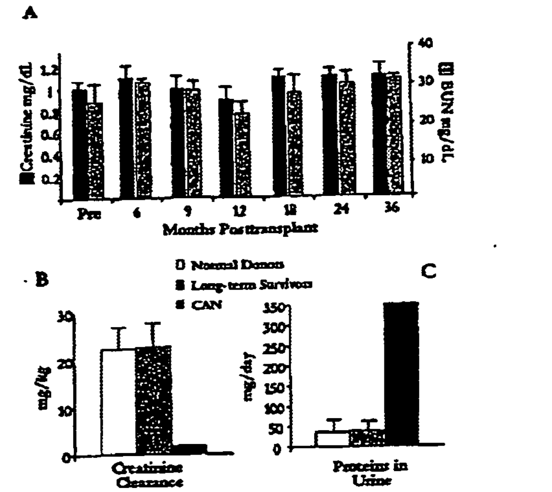

FIGURE 1. Normal kidney transplant function in LT survivors. Data are presented as pooled mean values ±SD from all the IT plus DSG-treated LT survivors (n=12) followed to the time limits indicated. Serum creatinine and BUN levels (A) were measured serially. Posttransplantation creatinine clearance (B) and urinary protein measurements (C) in LT survivors and in CAN were tested in 12 of 13 LT recipients (DAC excluded) within 3 weeks of the current survival times listed in Table 1. All the posttransplantation kidney function test values in tolerant recipients were consistently within the limits of healthy, uninephric donor controls (n=5).

We obtained detailed posttransplantation renal profiles for evidence of nephropathy. Data in Figure 1A show the values for pooled serum creatinine and BUN data from all the LT survivors followed up to 36 months after recipient nephrectomy. The data fell within the normal range compared with uninephric healthy donors (n=5). Creatinine clearance (Fig. 1B) and urinary proteins (Fig. 1C) in the LT survivors were similar to normal donor values but different from those of the IT-only treated recipient that developed CAN (Fig. 1, B and C). Absence of proteinuria in IT plus DSG-treated LT survivors is notable, because proteinuria is an early indicator of CAN in humans (*21*). Ultrasonographic follow-up confirmed normal renal function (data not shown). These data provide physiological evidence for stable, normal kidney transplant function in all 12 IT plus DSG-treated LT recipients.

In follow-up, the LT survivors demonstrated no overt loss of immune competence and had no opportunistic infections or neoplasia (*22*). This contrasts with reports of infection and ureteral fibrosis (*23, 24*) and lymphoid malignancies (*25, 26*) in other studies of highly immunosuppressed macaque recipients. Despite thorough T-cell depletion after IT treatment of our recipients, T-cell recovery restored an immunological

[5] Lobashevsky and Thomas. Manuscript in preparation.

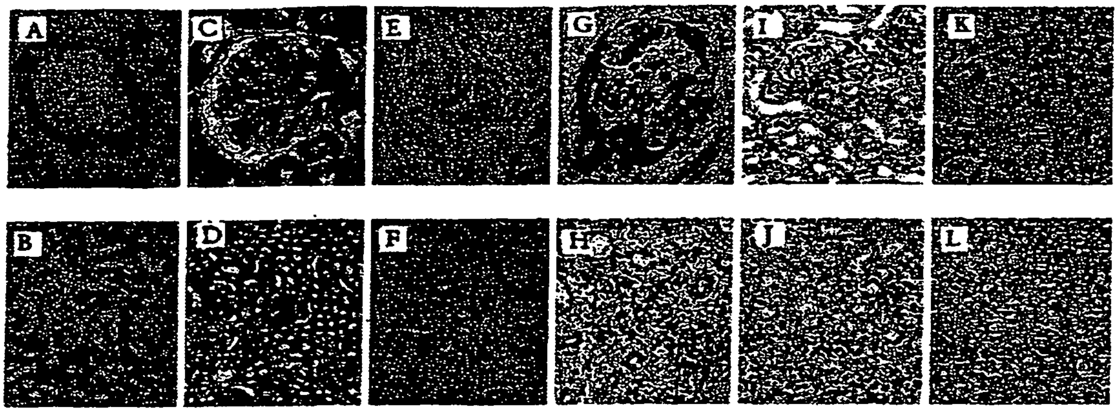

FIGURE 2. Absence of CAN in LT transplant biopsy specimens. Light microscopic analysis of hematoxylin and eosin-stained biopsies (×400) shows normal histology in representative LT survivors induced with IT and DSG, TLJ on day 850 posttransplantation [a (×40) and b (×200)], and T4P on day 920 [c (×40) and d (×200)]. The biopsy specimen from recipient 95C163, given IT only, shows severe interstitial fibrosis [e (×40)] and intimal proliferation [f (×100)]. Immunohistological analysis of TGF-β1 and FGF-2 expression in the CAN-positive biopsy specimen of 95C163 reveals prominent staining in glomeruli and interstitium, respectively [g (×40) and h (×200)]. Absence of TGF-β1 and FGF-2 in representative LT survivor AXE on day 740 [i (×40) and j (×200)]. Normal histology in the first transplant on day 1147 [k (×40)] and second transplant at day 447 [l (×40)], respectively.

balance of memory and naïve cells without generalized immune deficiency.[6]

Histological analysis confirms absence of CAN in LT survivors. Light microscopic analysis of early (30–55 days) and late (268–1147 days) biopsy specimens revealed uniform absence of interstitial cellular infiltration, fibrosis, intimal thickening, glomerulopathy, and tubular atrophy in all recipients induced with IT or F(Ab)$_2$IT plus DSG. Biopsy specimens from two LT representatives, TLJ (day 850) and T4P (day 920), are shown in Figure 2, A and B, C and D, respectively. Histopathological evaluations of all the LT kidney transplants were considered normal. In contrast, 95C163, the single recipient induced with IT only, exhibited abnormal renal allograft function and demonstrated histological evidence of all features of CAN (Fig. 2, E and F).

One hallmark of CAN is intragraft expression of cytokines and adhesion molecules, associated with early processes of graft destruction (*27*). Accordingly, we stained transplant biopsy specimens of stable LT survivors with mAbs to TGF-β1 and FGF-2, respectively. The chronically rejected transplant of recipient 95C163 served as a positive control. Figure 2, G and H, shows prominent staining for TGF-β1 and FGF in the glomeruli and interstitium, respectively, of this recipient's graft. In contrast, all stable transplants, represented here by recipient AXE at 740 days after transplantation (Fig. 2, I and J), were negative for both growth factors. Cytokine staining confirms that tolerant recipients did not develop CAN.

Specificity of kidney transplant tolerance. A claim of true immunological tolerance in LT transplant recipients should be supported by both the absence of CAN and in vivo evidence of donor-specific hyporesponsiveness. In vivo measurements of donor-specific immune tolerance include in vivo acceptance of donor skin grafts while rejecting MHC disparate third-party grafts, acceptance of a second kidney from the original donor without CI, and continued absence of antidonor Ab.

As previously reported, LT kidney transplant survivors, simultaneously given full thickness skin grafts from an unrelated third party and the living kidney graft donor, rejected the third-party grafts at 9.5±3.6 days (*15*). In contrast, donor-specific skin grafts were accepted without acute rejection for prolonged periods ($P<0.01$), confirming in vivo-specific antidonor hyporesponsiveness. Delayed rejection of the challenge donor skin grafts eventually occurred at a mean of 32±7 SD days but did not perturb the function of the tolerant kidneys. We, therefore, postulated that the late loss of donor skin was due to skin-specific minor antigens to which the recipient's T cells had not been tolerized, a phenomenon widely documented in other experimental models of tolerance (*28*). As an aside, the observation that donor skin grafts were eventually rejected attests to the rigorous immune competence of the LT tolerant recipients.

To further examine this premise, second kidney transplants from original donors were transplanted after rejection of donor skin grafts. Biopsy specimens of LT first and second kidney transplants revealed no evidence of CAN in either graft (Fig. 2, K and L, respectively). Survival of a histologically normal second kidney without immunosuppression represents a true test of tolerance that resists disruption by exposure to donor antigen-presenting cells present in the living donor-skin and second kidney grafts.

Engraftment of passenger hematopoietic cells after second kidney challenge. An unusual characteristic of this tolerance after IT plus DSG induction is a lack of demonstrable chimerism (*15*). Absence of microchimerism is postulated to reflect peritransplantation DSG inhibitory effects on NF-κB-dependent hematopoietic growth factors, endangering survival of passenger donor hematopoietic cells (*29*). Since LT survivors were receiving no immunosuppression, we rea-

[6] Hubbard et al. Manuscript submitted.

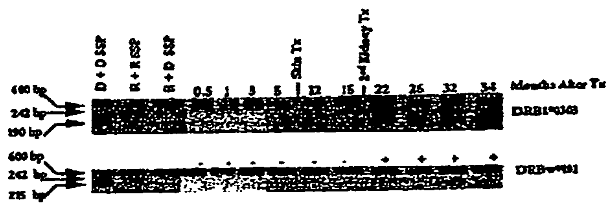

FIGURE 3. Stable microchimerism established after challenge with second donor kidney transplant at 2 years. Genomic DNA extracted from recipient AXE's peripheral blood was analysed in PCR-SSP using mismatched donor allele-specific primers. Horizontal arrows indicate sizes of internal control (γ-globin, 600 bp) and mismatched allele-specific products. Vertical arrows indicate the time of challenge donor and third-party skin and second donor kidney grafts, respectively.

soned that passenger cells in late second kidney transplants should engraft, if the recipients were truly tolerant of donor MHC antigens. The gel in Figure 3 confirms that premise, showing that previously undetected mismatched donor DRB alleles became consistently demonstrable in blood for >1 year after acceptance of the second kidney graft. Stable engraftment of donor hematopoietic cells from the second kidney transplant verified tolerance to donor MHC antigens.

Ab profile of tolerant recipients. Transplantation of allogeneic skin or kidneys in rhesus macaques elicits prominent antidonor IgG responses despite T-cell depletion (*30, 31*) or prolonged treatment with anti-CD40L (*32*). Our earlier studies in macaques induced with antithymocyte globulin and donor bone marrow suggested a temporal association between the appearance of antidonor IgG and chronic rejection (*30, 33*). We examined recipients' sera monthly for donor-reactive alloantibody by flow cytometry and found none of the LT tolerant monkeys exhibited IgG or IgM antidonor Abs at any time (Table 2). In contrast, all exhibited specific serum IgG Abs to some or all of the panel of antigens (diphtheria toxin, mouse IgG1, and FN18 IgG1 mAb [idiotope], and the environmental antigen Streptolysin O). The kinetics and magnitude of IgG responses to inoculated T cell-dependent and T cell-independent microbial antigens has also been found to be normal (Hubbard W, Thomas J, unpublished data). These results indicate intact humoral immunity and specific unresponsiveness to donor antigens, suggesting tolerance at both the T and B cell levels.

DISCUSSION

This report presents conclusive evidence in an outbred primate model that induction of true immune tolerance can reproducibly prevent both acute rejection and CAN in recipients with multiple, defined MHC class I and class II allele-mismatched kidney allografts. Immune tolerance was confirmed in vivo by multiple stringent criteria for which data are presented. These included: (1) indefinite graft acceptance in multiple recipients that maintained excellent graft function without CI; (2) specific and consistent absence of antidonor Ab, despite normal Ab responses to unrelated antigens; (3) histological exclusion of cellular rejection and CAN; (4) stable engraftment of donor hematopoietic cells from the donor's second transplant; and (5) indefinite acceptance of a second kidney from the donor without immunosuppression, the ultimate standard of tolerance (*34*).

As previously reported, primary exposure to passenger cells in first transplants during immunosuppressive induction did not result in demonstrable chimerism (*15*). In contrast, we show here that hematopoietic passenger cells from second kidney grafts established stable engraftment without immunosuppression. This observation confirms the validity of our initial chimerism PCR-SSP test results indicating a lack of chimerism after IT plus DSG induction, putatively due to DSG's inhibition of NF-κB-related growth factor effects. More importantly, however, these data support a claim for specific tolerance to both donor kidney and hematopoietic cells. We conclude that protracted rejection of donor skin, consistently observed in this model, is probably due to minor skin-specific antigens to which the recipients were not previously exposed.

Success in nonhuman primate tolerance without adverse side effects is the gold standard for human application. How-

TABLE 2. Antibody response profiles in tolerant rhesus macaque kidney transplant recipients

Recipient	Anti-donor IgM Ab[a]	Anti-donor IgG Ab[a]	Anti-DT OD[b,c]	Anti-mouse IgG OD[b,d]	Anti-FN18 OD[b,d]	Anti-StO OD[b,e]
TLJ	Negative	Negative	0.123	0.034	0.129	0.010
T4P	Negative	Negative	0.186	0.220	0.463	0.356
AXE	Negative	Negative	0.208	0.147	0.225	0.111
95C137	Negative	Negative	0.093	0.108	0.231	0.189
CFB	Negative	Negative	1.141	0.738	0.825	0.743
CHE	Negative	Negative	0.142	0.143	0.144	0.441
DAC	Negative	Negative	0.110	0.177	0.337	0.365
96C176	Negative	Negative	0.519	0.195	0.349	0.009
96C044	Negative	Negative	1.025	0.125	0.175	0.082
95D471	Negative	Negative	0.501	0.197	0.358	0.409
97D141	Negative	Negative	0.172	0.050	0.050	0.115
97D389	Negative	Negative	0.767	0.473	0.676	0.784
95C163	Negative	Negative	0.105	0.010	0.096	0.200

[a] Antidonor Ab binding measured by flow.
[b] Measured by ELISA, OD_{405} corrected by background subtraction.
[c] Normal range: none exceeded 0.045 OD (n=8).
[d] Normal range: none exceeded 0.028 OD (n=8).
[e] Normal range: 0.011–1.23 OD (n=8).

ever, a firm definition of stable primate tolerance has been difficult. Early studies commonly demonstrated a state of split tolerance (20; 23; 30; 32; 33) in which most recipients developed antidonor IgG in vivo, despite prolonged graft acceptance and in vitro donor-directed T-cell hyporesponsiveness. Of note, split tolerance with persistent antidonor Ab has also been observed in LT recipients after induction with anti-CD40L (32) or IT without DSG (*31*). In our experience and that of others, LT survivors with split tolerance develop CAN and subsequent graft loss at 3 months to 4 years. No previously reported macaque allograft series has documented true MHC tolerance that includes B cell tolerance, absence of CAN, and indefinite acceptance of challenge second donor renal grafts and donor hematopoietic cells. In a recently updated series of 13 cynomolgus macaques induced by a combination of antithymocyte globulin, total body irradiation, thymic irradiation, splenectomy, donor bone marrow infusion, and 4 weeks of cyclosporine, kidney allografts survived a median 200 days with two stable long survivors (*24*). Development of antidonor Ab was infrequent in that study, but the specificity of humoral unresponsiveness was not determined. Here we report specific humoral unresponsiveness in a large series of tolerant recipients given only IT plus DSG. The findings are consistent with the concept that donor-specific T cell and B cell tolerance is linked to stable tolerance without CAN in MHC-mismatched rhesus macaques.

A relevant clinical issue is the availability of a surrogate test for tolerance. We observed that histology and function of the first kidney allograft were unaffected by the protracted rejection of a challenge donor skin graft. Since the second kidney was subsequently accepted, this casts doubt about the validity of permanent donor skin acceptance as a criterion of kidney transplant tolerance. Our observations suggest possible correlates of stable kidney transplant tolerance. These would include a combination of (1) donor-specific B cell tolerance and (2) stable engraftment of infused donor hematopoietic stem cells in recipients previously negative for chimerism.

The absence of TGF-β1 and FGF-2 expression in LT tolerant transplant biopsy specimens is consistent with freedom from CAN, since the presence of these growth factors associates with human CAN (*35*). In clinical transplantation, development of CAN after acute rejection suggests immune-mediated damage so intense as to escape restraint by modern immunosuppressive drugs (*36*). Recently, a multifactorial concept of CAN has emerged (*2–4*). Herein, nonimmune donor graft injury/dysfunction from ischemia-reperfusion, brain death, and even donor age is compounded by inflammation, enhancing alloimmune recognition. While the discovery of nonimmunological factors in CAN is essential for fully understanding the pathogenesis, numerous observations highlight the pivotal role of allogeneic factors (*6, 37–39*). In addition to preceding acute rejection episodes (*4*), preformed Abs and degree of HLA mismatch (*36, 40*) are predictive of CAN, consistent with alloimmunity as a final arbiter of CAN. Extending this logic, tolerant monkeys without cellular or humoral allograft reactivity in the absence of CI should be protected from CAN. The results suggest this to be correct.

Our approach to primate tolerance without CAN takes advantage of a novel synergy between two unusual drugs, DSG and IT (*16*). While the molecular basis of this synergy remains to be proven, there are several points worth emphasizing. First, IT, from intact IgG or $F(Ab)_2$, is particularly effective in abolishing T cells (*41*). No other agent so profoundly eliminates lymph node T cells (*16*), the major site of T cell-DC interaction. $F(Ab)_2$IT seems to have an advantage of more uniform tolerance results, possibly related to reduction of proinflammatory cytokine responses in vivo (*15*). However, neither $F(Ab)_2$IT alone nor DSG alone have reproducibly promoted tolerance (*14, 15*). Thus, contrary to our earlier perspective, tolerance after IT induction is not merely a matter of controlling proinflammatory cytokine responses (*14*).

DSG-induced maturation arrest of DC during early T-cell depletion may be involved mechanistically in the lack of alloantibody and CAN. Immature DC only process antigen, whereas mature DC only present the processed antigen to T cells or B cells (*42*). The DSG blockade of DC maturation is presumably via an NF-κB-dependent process (*43, 44*). The brief time required for DSG treatment may be explained by the limited lifespan of myeloid DC. While mature donor DC precursors directly present donor alloantigens, they constitute a short-lived, nonrenewable component unless hematopoietic chimerism is established, which is not the case after induction with IT plus DSG.

Conceivably, early DC presentation of donor alloantigens, either via the direct or indirect pathway (*45*), is virtually blocked by peritransplantation DSG administration. This attenuation of alloantigen presentation via DC to newly reconstituted T cells is likely to persist because the peak window of alloantigen processing lapses with healing of the graft, and cytokine "danger" signals (*17*) leading to DC maturation decrease after inflammation from the surgical procedure subsides. We suggest the net result is a blockade of reconstituted T-cell activation by alloantigen in the face of alloantigen persistence, conditions that favor establishment of true T and B cell tolerance without CAN (*46*).

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